SYNOPSIS

Mitochondria are essential eukaryotic *organelles*, acting as the sites for numerous crucial metabolic and signalling pathways. The biogenesis of mitochondria requires efficient targeting of several hundreds of proteins from the cytosol, to their varied functional locations within the *organelle*. The translocation of localized proteins across the inner membrane, and their subsequent folding is achieved by the ATP-dependent function of mitochondrial Hsp70 (mtHsp70). It is a bonafide member of the Hsp70 chaperone family, which are involved in a multitude of functions, together aimed at protein quality control and maintenance of cellular homeostasis. These varied functions of Hsp70 proteins require binding to exposed hydrophobic patches in substrate polypeptides thus preventing non-productive associations. The interaction with substrates occurs through the substrate-binding domain (SBD) and is regulated by the ATPase activity of the nucleotide-binding domain (NBD), through a series of conformational changes. Conversely, substrate binding to the SBD also stimulates ATP hydrolysis, and thereby the core activities of the two domains are regulated by mutual allosteric signalling. This mechanism of bidirectional inter-domain communication is indispensable for Hsp70 function, which is characterized by cycles of substrate binding and release, coupled to cycles of ATP binding and hydrolysis. The process of allosteric regulation in Hsp70 proteins has been comprehensively investigated, especially in the bacterial homolog, DnaK. However, the *in vivo* functional significance of inter-domain communication in the eukaryotic mtHsp70 system and the mechanism of its regulation remain unexplored. Furthermore, the complex physiological implications of impairment in allosteric communication and their correlation with diverse disease conditions, including Myelodysplastic syndrome (MDS), and Parkinson's disease (PD), are yet to be elucidated.

Based on this brief introduction, the primary research objectives set out in the present thesis were to:

1. uncover the regulation of ligand-modulated allosteric communication between the two domains of mtHsp70; and its *in vivo* significance in the context of protein import into the *organelle*. **(Chapter 2)**

2. understand the role of mtHsp70 in progression of Parkinson's disease; and to study the modulation of α-synuclein toxicity by the protein quality control function of the mtHsp70 chaperone network. **(Chapters 3 and 4)**

We have employed a battery of genetic and biochemical approaches to investigate the above questions using the *Saccharomyces cerevisiae* mtHsp70 protein, Ssc1; an essential protein that is involved in a plethora of critical functions in this eukaryotic model system.

Objective 1: Structural studies, primarily in bacterial DnaK, have yielded mechanistic insights into its interactions with ligands and cochaperones, as well as conformational transitions in different ligand-bound states. In recent years, the availability of crystal structures of full-length DnaK and detailed information from NMR studies and single-molecule resolution spectroscopic analyses (both DnaK and eukaryotic Hsp70s), have significantly contributed to our understanding of the inter-domain interface, critical residues and contacts, and the energetics of the entire process of ligand-modulated conformational changes. Although eukaryotic mtHsp70s have a high degree of conservation with DnaK, they possess significant differences in their conformational and biochemical properties. They are essential for a vast repertoire of physiological functions, which are distinctly different from their bacterial counterpart. Using a combined *in vivo* and *in vitro* approach, we have uncovered specific structural elements within mtHsp70s, which are required for

allosteric modulation of the chaperone cycle and maintenance of *in vivo* functions of the protein. Foremost, we demonstrate that a conserved SBD loop, $L_{4,5}$ plays a critical role in inter-domain communication, and multiple mutations in this loop result in significant growth and protein translocation defects. The mutants are associated with a specific set of altered biochemical properties, which are indicative of impaired inter-domain communication. Using the loop $L_{4,5}$ mutant, E467A as a template for genetic screening, we report a series of intragenic suppressor mutations, which are capable of correcting a distinct subset of the altered properties, and thereby leading to restoration of *in vivo* functions, including growth, preprotein import and mitochondria biogenesis. The suppressors modify the altered conformational landscape associated with E467A, and also provide us with information regarding unique aspects governing the regulation of allosteric communication, especially in physiological contexts. Strikingly, they reveal that restoration of communication in the NBD to SBD direction is sufficient for function, when the protein is primed in a high ATPase activity state. In this unique scenario, the requirement for ATPase stimulation upon substrate binding is rendered unnecessary, thereby making conformational changes in the SBD to NBD direction, dispensable for function. Further, we provide evidence to show that loop $L_{4,5}$ functions synergistically with the linker region, working in tandem for organization of the inter-domain interface and propagation of communication. Together, our analyses provide the first insights into regulation of allosteric inter-domain communication *in vivo* and their implications in mitochondrial protein translocation and *organelle* biogenesis.

Objective 2: Point mutations in the loop $L_{4,5}$ have been associated with Myelodysplastic syndrome. Additionally, a mutation isolated in clinical cases of Parkinson's disease was found to be impaired in allosteric communication. These observations further highlight the importance of efficient inter-domain communication in mtHsp70 in the complex

physiological scenario of eukaryotic cells. Independent clinical screens of PD patients have revealed unique point mutations in the mtHsp70 and a strong association of the gene locus with the disease progression. This is also correlated with decreased mtHsp70 levels in affected neurons and the interactions of this protein with established PD-candidate proteins like α-synuclein and Dj-1. Further, mitochondrial dysfunction is a common phenomenon associated with neurodegenerative disorders. To understand the specific role of mtHsp70 in PD, we have developed a yeast model for studying the disease variants in isolation from other players of the multifactorial disease, and in complete absence of the wild type protein. We generated two analogous PD-mutations in Ssc1, R103W and P486S; which recapitulated the symptoms of mitochondrial dysfunction in affected neurons, including cell death, inner membrane depolarization, increased generation of ROS, and respiratory incompetence. At the molecular level, we observed an increased aggregation propensity of R103W, while P486S exhibited futile enhanced interaction with J-protein cochaperone partners thereby resulting in loss of chaperoning activity and impaired mitochondrial protein quality control. Remarkably, these altered biochemical properties mimicked similar defects in the human mtHsp70 variants, therefore, affirming the involvement of mtHsp70 in PD progression.

To further investigate the relevance of impaired mitochondrial protein quality control in PD, we have explored whether mtHsp70 can act as a genetic modifier of α-synuclein toxicity. It is known that α-synuclein can act as an unfolded substrate for the Hsp70 chaperone system and also deposits as intracellular aggregates in PD-affected brains. Intriguingly, it is known to translocate into mitochondria under conditions of neuronal stress in spite of lacking a canonical mitochondrial signal sequence. Utilizing our yeast-PD model, we find that targeting of α-synuclein A30P disease variant into mitochondria leads to a severe mitochondrial dysfunction phenotype in the wild type Ssc1 background, but not the P486S mutant background. This results in multiple cellular manifestations, which are

reversed upon overexpression of the Ssc1 chaperone. Significantly, increasing the J-protein cochaperone availability also leads to reversal of the mutant-associated defects. However, the simultaneous overexpression of both together does not additively improve the protective effects; highlighting the importance of the relative availability of chaperone and cochaperone proteins in preventing aggregation. Our analyses further reveal that while both the wild type and P486S Ssc1 proteins are equally capable of delaying aggregation of α-synuclein, only the wild-type chaperone is better able to prevent aggregation in the presence of its J-protein cochaperone, leading to accumulation of soluble oligomeric species. These observations raised the intriguing possibility, that the reduced chaperoning ability of the proline to serine PD-mutant is, in fact, a compensatory adaptation, favoring the aggregation of α-synuclein over its more toxic soluble oligomeric form. We verify this hypothesis with the aggregation kinetics of A30P α-synuclein, whose intrinsically lower aggregation tendency results in a pronounced delay in aggregation with the wild-type chaperone, thereby strongly favoring the toxic oligomeric species and correlating with the observed lethality in yeast cells. In conclusion, our study provides a model of α-synuclein aggregation-related toxicity and its modulation by the extent of protein quality control within the mitochondrial matrix, through the action of the mtHsp70 chaperone network.